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Proportion of ovine pulmonary adenocarcinoma in Danish sheep at slaughter

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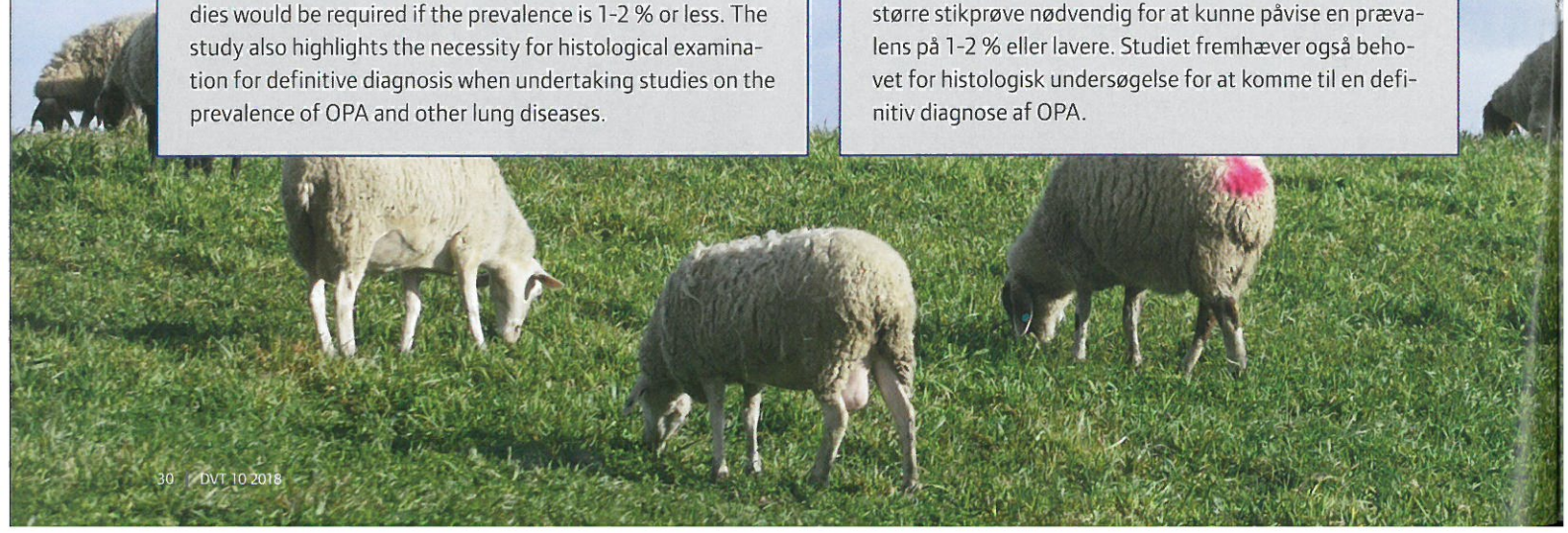
Summary

Ovine pulmonary adenocarcinoma (OPA) is prevalent in many sheep producing countries such as the United Kingdom, but the prevalence of OPA is not known in Denmark, where sheep production is less intensive. Our objective was to estimate the proportion of OPA in Danish sheep at slaughter. All adult sheep (n=235) and lambs (n=1245) slaughtered at the largest Danish sheep abattoir, receiving animals from the entire country, were collected from 29th February to 25th April 2016 in a cross-sectional-like study. A total of 1480 pairs of lungs from lambs and adult sheep were examined visually and by palpation. Based on gross examination, 25 animals were suspected to have OPA and were examined in more detail by histology and immunohistochemistry. None had OPA. This is in contrast to previous incidental findings of OPA at two farms in 2014. Based on the sample size of adult sheep examined, the prevalence should not exceed 1.3 % if the samples collected were representative of the Danish sheep population. However, for greater accuracy, larger studies would be required if the prevalence is 1-2 % or less. The study also highlights the necessity for histological examination for definitive diagnosis when undertaking studies on the prevalence of OPA and other lung diseases.

Sammendrag

Lungeadenomatose (ovine pulmonary adenocarcinoma (OPA)) forekommer hyppigt i nogle lande med stor fåreproduktion såsom Storbritannien, men forekomsten i Danmark er ikke kendt. Målet med vores studium var at estimere andelen af får med OPA ved slagtning. Alle voksne får (n=235) og lam (n=1245), som blev slagtet på det største danske fåreslagtehus, der modtager får fra hele landet, i perioden 29. februar til 25. april 2016 blev inkluderet i studiet. Lungerne fra får og lam blev undersøgt visuelt og ved palpation, og på baggrund af den makroskopiske undersøgelse blev 25 dyr mistænkt for at have OPA. Disse blev undersøgt nærmere med histologi og immunohistokemi, hvorved alle blev vurderet til at være fri for OPA. Dette står i kontrast til en tidligere rapport om fund af OPA i to danske fårebesætninger i 2014. På baggrund af stikprøvestørrelsen for voksne får vurderes det, at selvom OPA kan være til stede i Danmark, så forventes prævalensen ikke at overstige 1,3 %. Dog er en større stikprøve nødvendig for at kunne påvise en prævalens på 1-2 % eller lavere. Studiet fremhæver også behovet for histologisk undersøgelse for at komme til en definitiv diagnose af OPA.



Introduction

Ovine pulmonary adenocarcinoma (OPA, also known as jaagsiekte, ovine pulmonary carcinoma and sheep pulmonary adenomatosis) is a contagious neoplastic ovine lung disease caused by infection with jaagsiekte sheep retrovirus (JSRV) (1). The virus primarily infects sheep, but can also cause subclinical infections in goats (2, 3). OPA is commonly seen in sheep of three to four years of age (4,5,6) and is characterized as a respiratory wasting disease where the virus induces neoplastic transformation in type II pneumocytes and Club cells (Clara cells) (7). This results in tumour growth which may culminate in replacement of a large proportion of the normal lung parenchyma which impairs normal lung function. The lack of lung function and/or secondary bacterial infections commonly associated with OPA are invariably fatal in sheep (4). Classical forms of the JSRV infection result in progressive respiratory distress resulting in death, but atypical forms also exist where the tumours are only detected at necropsy. Classical and atypical cases can be differentiated through necropsy where atypical cases appear without lung fluid, the tumour nodules are firm and dry, and histologically there are fewer neutrophils and macrophages but increased proliferation of fibroblasts (8).

OPA is present in most sheep rearing countries worldwide, except Australia and New Zealand, and was eradicated from Iceland in 1952 (9-11). Farmers in the UK have reported annual losses of up to 20 per cent due to OPA in their flocks (12) and losses of 30 to 50 per cent in flocks were reported in Iceland in the 1930s during an outbreak of dual infection with maedi-visna virus and OPA (4). As OPA becomes endemic in a flock the mortality rate frequently drops to 1-5 per cent (12), but sheep welfare and farming profitability are still adversely affected.

Serological diagnosis of OPA is not possible because a humoral immune response to the virus is not evident (13-14). Furthermore, clinical signs are absent in the early stages of the disease (15). Definitive diagnosis of OPA is dependent on gross post-mortem examination, followed by histology and, in a small number of equivocal cases, specific immunohistochemistry (15-16), while PCR is deemed to have a sensitivity of around 0.11 only (17).

OPA has been detected previously, based on histological lesions combined with PCR (diagnosed by Prof. M. Ganter, University of Veterinary Medicine Hannover, Germany), as an incidental finding in sheep from two farms in Southern Jutland, Denmark during a study to determine the prevalence of maedi-visna virus (18), but the prevalence of OPA in Denmark has never been reported. The aim of the present study was to estimate the proportion of Danish sheep with OPA at slaughter based on gross post-mortem examination followed by histology and immunohistochemistry of lungs from sheep and lambs with suspicious gross lesions at the largest ovine abattoir in Denmark.

Materials and methods

Study design and sample size

The Danish sheep population is comprised mostly of Texel crosses and the study was an observational cross-sectional study based on the sheep and lambs slaughtered at the largest Danish sheep abattoir, located in the Southern part of Jutland, Denmark. This population was deemed useful because individual animal identification was possible for adult animals. This abattoir slaughtered approximately 10,000 of the 80,000 sheep, lambs and goats slaughtered in Denmark in 2015, and the animals were from all parts of the country. All sheep and lambs slaughtered from the 29th of February to the 25th of April 2016 were included in this study. The lambs brought to slaughter at this time of year were born in spring 2015 and so were a minimum of ten months old. Adult sheep were the primary age group of interest, as OPA is most prominent in adult animals of three to four years of age (4, 12, 16), but all ages were examined.

A minimum sample size of 203 adult sheep was required based on the standard sample size estimator for proportions (19), a maximum allowable error of 0.03, a reported infection prevalence estimate of 10 per cent from a study in Scotland (17) and a clinical disease prevalence of 5,6 % estimated in a study of fallen stock in the North-east of England (20).

Gross examination and sample collection

Sheep and lambs were slaughtered by electrical stunning followed by immediate exsanguination. Heads were removed from sheep older than one year due to legislation with respect to Transmissible Spongiform Encephalopathy. Lungs, heart and liver remained attached to the carcass hanging from the trachea. One person (CE) examined all the lungs and carcasses approximately four hours after the slaughter of all animals had taken place each day. Gross visual examination and palpation of the lungs were used to identify any lesions on the dorsal and ventral surfaces. Palpation was used to identify nodular lesions or tumours of classical or atypical OPA type in the deeper tissue. Additionally, lesions consistent with pleurisy, abscesses, necrosis, fibrosis and verminous pneumonia were noted along with their locations. All cases with any gross lesions were initially classified as »suspect OPA«, while those with no gross lesions were categorised as OPA negative. Samples of »suspect OPA« lung >

tissue, approximately 2 cm x 1.5 cm x 0.5 cm, were placed into 10 % neutral-buffered formalin solution for histological examination and immunohistochemistry, and maedi-visna virus PCR if the latter was suspected after histological examination.

Histological Examination and Immunohistochemistry

Tissue samples were processed routinely through graded alcohols and embedded in paraffin wax. Sections (4µm) were mounted on charged glass slides (Superfrost Plus™, Menzel-Glaser, Germany) and stained with H&E (21). Semi-serial sections were subjected to immunohistochemistry (IHC) for the JSRV envelope protein (envJSRV) (22) as described previously (23). Briefly, tissue sections were dewaxed in xylene, rehydrated through graded alcohols to water, placed in 3 % hydrogen peroxide in methanol (v/v) for 20 minutes at room temperature (RT) to block endogenous peroxidase activity and then washed in tap water. For antigen retrieval, slides were placed in 0.01M citrate buffer (pH 6) in an autoclave for 10 minutes at 121°C. The slides were left to cool to 50°C, washed in running tap water and then placed in immunostaining racks (Shandon™ *Sequenza*™) and immersed in phosphate buffered saline containing 0.05% Tween 20 (PBST). Non-specific antibody binding was blocked by incubating the slides with 25 % normal goat serum in PBST at RT for 30 minutes. Anti-JSRV Env mouse monoclonal antibody (22) was applied at 1:150 dilution in PBST. Normal mouse IgG, diluted at 1:150 in PBST, was applied to duplicate semi-serial sections as negative control preparations, and sections from a natural case of OPA were used as a positive control. All slides were incubated overnight at 4°C then washed in PBST three times. Bound primary antibody was visualised using a proprietary goat anti-mouse HRP polymer (Envision™ System-HRP, Dako, Ely, UK) applied for 30 minutes at RT as per manufacturer's instructions. The chromogen 3'-diaminobenzidine tetrachloride (DAB) (Envision™ System-HRP) was applied for 10 minutes, and slides were subsequently washed in tap water, stained with haematoxylin »Z« (Cellpath Ltd, Powys,UK), »blued« in Scott's tap water substitute, dehydrated through graded alcohols, cleared in xylene and mounted using Consul-mount™ (Thermo Shandon Ltd, Runcorn, UK). Any suspect OPA cases that were positive on histological examination and/or immunohistochemistry were definitively diagnosed as OPA and were thus the case definition. Any cases considered suspicious for maedi-visna virus (MVV) infection were also subject to a specific PCR.

PCR for MVV

DNA was isolated from formalin-fixed, paraffin-wax embedded (FFPE) lung tissue samples from suspected cases using the RecoverAll™ Total Nucleic Acid Isolation Kit (Invitrogen) as described in the manufacturer's instructions. MVV was detected by semi-nested PCR targeting the *gag* gene as described previously (24).

Statistical Analyses

A post-hoc sample size calculation was performed to estimate the maximum prevalence given a negative result in the sample of adult sheep (19), assuming a population size of approximately 92,800 sheep in Denmark and a confidence interval of 95 % in detecting OPA affected sheep (S. Kobberøe, SEGES, Aarhus, Denmark, personal communication 2016).

Results

The lungs from 1245 lambs and from 235 adult sheep over the age of one year were examined. They originated from 134 different farms, mainly from Jutland. Twenty-five (8 lambs and 17 adults) of the 1480 sheep were categorized as suspect OPA cases by gross examination of the lungs (Table 1). The suspect OPA lungs were mostly enlarged and heavy, consistent with the gross description of classical OPA. Lesions were purple or grey in colour and focal or multi-focal coalescing throughout the lung lobes. Seven of the suspected OPA cases showed gross lesions of chronic suppurative pneumonia as denoted by fibrous adhesions and abscesses. Normal sized lungs with smaller lesions, frequently on the diaphragmatic lobes, not typical of classical OPA or parasitic pneumonia were also sampled for histological examination. Parasitic pneumonia (minor to major lungworm infestation) was observed in 17 % of lambs (214/1245) and 21% of adult sheep

Table 1. Results from gross examination at slaughter of lungs of sheep at a Danish abattoir in February -April 2016

Sheep Age	No. examined	Parasitic pneumonia	Bacterial pneumonia	Suspected OPA	Confirmed OPA
<1yr	1245	214 (17 %)	9 (0.7 %)	8 (0.6 %)	0
> 1yr	235	50 (21 %)	5 (2 %)	17 (7 %)	0

(50/235). Bacterial pneumonia was diagnosed in 0.7 % of lambs (9/1245) and 2.0 % of adult sheep (5/235).

As summarised in Table 2, histological examination and IHC showed that none of the 25 suspected cases had OPA, instead maedi-visna infection, parasitic pneumonia or bacterial pneumonia were detected, or no pathological changes were found. Maedi-visna virus infection was diagnosed in 11 (65 %) of the 17 »OPA suspected« adult sheep by histological examination of H&E stained slides. These findings were confirmed by a semi-nested PCR for detection of MVV. Three lambs and three adults in this group had parasitic lesions, two lambs and one adult had lesions typical of bacterial infection, and one ewe had lesions suggestive of cor pulmonale. Three lambs and one adult were devoid of histological lesions suggesting that the observed gross lung lesions were acute post-mortem changes, possibly associated with the method of slaughter.

Based on the negative finding for OPA in all 1245 lambs and 235 adult sheep examined, a maximum prevalence of OPA in the Danish sheep population was estimated at 1.3 % by the post-hoc sample size calculation.

Discussion

This is the first study to estimate the proportion of OPA in sheep at abattoirs in Denmark. No cases were found, but despite the negative findings two previous incidental findings of OPA, both definitively diagnosed by PCR (18), showed that OPA is present in sheep in Denmark. Given the number and age distribution of the sheep examined, the maximum prevalence of OPA in sheep slaughtered at this large Danish abattoir was estimated to be 1.3 %.

The present study was designed to detect OPA based on a 5.6 % prevalence found in fallen stock in the North-east of England (20). However, the limit of detection for the study was reduced to 3 % because the majority of clinical OPA cases are either euthanized or die on farm. This supposition is supported by a similar abattoir study in Birmingham, receiving sheep from throughout the United Kingdom, that examined the lungs of 3385 adult sheep in which 31 animals (0.9 %) were definitively diagnosed with OPA based on gross examination, histology and IHC (24). An abattoir study in Spain reported a similar prevalence of OPA, 1.3 % (21/1600), in sheep over two years of age in Zaragoza (8) using the same diagnostic methods. However, in that study both atypical (n=11) and classical (n=8) cases of OPA and two sheep with mixed forms were reported, while the UK study found only classical OPA. An even lower prevalence of 0.02 % (52 sheep from a total of 280,000 slaughtered during 1964) was reported from an abattoir in Edinburgh, Scotland, UK (26). However, the latter study used gross examination of the lungs only and, as highlighted in the present study, this probably misclassified an unknown number of cases where histology and IHC would have made definitive diagnoses. In addition, the age of the sheep was not reported, and it is highly likely that primarily lambs under one year of age were examined (25). This renders invalid any comparison of this early study with the other studies which focused on sheep older than one year of age.

Lambs with OPA, although uncommon, have been reported previously (1, 12, 27, 28), which is why we also examined this age group. However, despite gross examination of the lungs in the present study being conducted by one person, to exclude inter-observer variation, early and/or very small OPA lesions could easily be undetected by this procedure. Therefore, we have not put any emphasis on the lack of OPA in the lambs in the post-hoc maximum prevalence calculation as this would have resulted in a much lower, and probably less accurate, maximum prevalence.

Considering the 235 adult animals only, the lowest proportion of affected animals in the population which would have a 95 % probability of resulting in one or more positive OPA cases in this sample size is estimated at 1.3 (19). Therefore, we cannot say from our findings that the population is negative, but we can predict that the prevalence will be low (1.3 % or less) which is similar to the prevalence reported previously in the UK (25) and in Spain (8). Therefore our results, in combination with the previous Danish report (18), indicate that OPA is present in Denmark but at a relatively low level.

Table 2. Results of histological examination of sheep lungs selected due to gross lesions as suspected ovine pulmonary adenocarcinoma at a Danish abattoir in February – April 2016.

Age	No. histology	Maedi visna	Parasitic pneumonia	Bacterial pneumonia	Other	No significant finding
<1year	8	0	3	2	0	3
> 1yr	17	11	3	1	1§	1

§cor pulmonale

This study took place during March and April 2016, and lambing in Denmark takes place mainly from March to May. Therefore, the lambs slaughtered during the study period were 10-12 months of age, and the adult sheep would have been mainly ewes that were not in lamb or had problems related to reproduction whereas unhealthy, older animals are usually culled in autumn and winter. Therefore, the time of year may have reduced the probability of finding OPA positive adults.

Despite the results of this study, suggesting a low proportion of OPA in Danish sheep at slaughter in Denmark, the potential adverse economic and welfare impact of the disease is a cause for concern and monitoring for OPA should continue.

In conclusion, this study did not find OPA in the sheep and lambs examined at an abattoir in Jutland, Denmark, despite the disease having been reported previously in two sheep farms in the area. The present study was limited by the restricted number of adult sheep available for examination, the use of animals from one slaughterhouse only, the time of year of sampling and the lack of fallen stock and animals with respiratory disease available to examine. Further studies should address all of these factors and must include definitive diagnosis of OPA by histological examination supplemented with IHC in equivocal cases.

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Referencer

1) Palmarini M, Sharp J, De las Heras M: Jaagsiekte Sheep Retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. J. Virol. 1999, 73, 6964–6972.
2) Rajya BS, Singh CM: Pathology of pneumonia and associated respiratory disease of sheep and goats. 1. Occurrence of jaagsiekte and maedi in sheep and goats in India. Am. J. Vet. Res. 1964, 25, 61–67.
3) Sharp JM, Angus KW, Jassim FA, Scott FM: Experimental transmission of sheep pulmonary adenomatosis to a goat. Vet. Rec. 1986, 119, 245.
4) Dungal N, Gislason G, Taylor EL. Epizootic adenomatosis in the lungs of sheep—comparisons with Jaagsiekte, verminous pneumonia and progressive pneumonia. J. Comp. Pathol Therap. 1938, 51, 46–68.
5) Hunter AR, Munro R: The diagnosis, occurrence and distribution of sheep pulmonary adenomatosis in Scotland 1975 to 1981. Br. Vet. J. 1983, 139, 153–164.
6) Gonzalez L, Juste RA, Cuervo LA, Idigoras I, Saez de Ocariz C: Pathological and epidemiological aspects of the coexistence of maedi-visna and sheep pulmonary adenomatosis. Res Vet Sci. 1993, 54, 140–146.
7) Platt JA, Kraipowich N, Villafane A, DeMartini JC: Alveolar Type II cells expressing Jaagsiekte Sheep Retrovirus capsid protein and surfactant proteins are the predominant neoplastic cell type in ovine pulmonary adenocarcinoma. Vet. Pathol. 2002, 39, 341–352.
8) De Las Heras M, Calafat JJ, Jaime JM, Garcîa de Jalón JJ, Ferrer LM, Goti M, Minguijon E: Sheep pulmonary adenomatosis (jaagsiekte) in slaughtered sheep. Variation in pathological characteristics. Med. Vet, 1992, 9 (Suppl), 52–53.
9) Palsson PA: Maedi/visna of sheep in Iceland. Slow viruses in sheep, goats and cattle, Reykjavik, 13-14 July 1982, p. 3-20. Commission of the European Communities, 1985.
10) Demartini JC, Rosaido RH, Lairmore MD: The etiology and pathogenesis of ovine pulmonary carcinoma (sheep pulmonary adenomatosis). Vet. Microbiol. 1988, 17, 219–236.
11) Salvatori D, De las Heras M, Sharp JM: Ovine pulmonary adenocarcinoma: The story to date. In Practice 2004, 26, 387–392.
12) Sharp JM, DeMartini JC: Natural history of JSRV in sheep. Curr. Top. Microbiol. Immunol. 2003, 275, 55–79.
13) Ortín A, Minguijon E, Dewar P, Garcia M, Ferrer LM, Palmarini M, Gonzalez L, Sharp JM, De las Heras M: Lack of a specific immune response against a recombinant capsid protein of Jaagsiekte Sheep Retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. Vet. Immunol. Immunopathol. 1998, 61, 229–237.
14) Summers C, Neill W, Dewar P, Gonzalez L, van der Molen R, Norval M, Sharp JM: Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma. J. Gen. Virol. 2002, 83, 1753–1757.
15) Cousens C, Graham M, Sales J, Dagleish MP: Evaluation of the efficacy of clinical diagnosis of ovine pulmonary adenocarcinoma. Vet. Rec. 2008, 162, 88–90.
16) Scott PR, Griffiths D, Cousens C: Diagnosis and control of ovine pulmonary adenocarcinoma (Jaagsiekte). In Practice 2013, 35, 382–397.
17) Lewis FI, Brülisauer F, Cousens C, McKendrick IJ, Gunn GJ: Diagnostic accuracy of PCR for Jaagsiekte Sheep Retrovirus using field data from 125 Scottish sheep flocks. Vet. J. 2011, 187, 104–108.
18) Hartmann A: Maedi-visna hos væddere i danske fårebesætninger (Maedi-visna in rams in Danish sheep flocks). Veterinary Master Thesis, Department of Large Animal Sciences, University of Copenhagen, Frederiksberg, Denmark, 2014.
19) Houe H, Ersbøll AK, Toft N: Introduction to Veterinary Epidemiology, Biofolia, Frederiksberg, Denmark, 2004, pp. 112–115.
20) Lovatt FM, Strugnell BW: An observational study involving ewe postmortem examination at a fallen stock collection centre to inform flock health interventions. Vet. Rec. 2013, 172, 504.
21) Feldman AT, Wolfe D: Tissue processing and hematoxylin and eosin staining. Methods Mol. Biol. 2014, 1180, 31–43.
22) Wootton SK, Metzger MJ, Hudkins KL, Alpers CE, York D, DeMartini JC, Miller AD: Lung cancer induced in mice by the envelope protein of Jaagsiekte Sheep Retrovirus (JSRV) closely resembles lung cancer in sheep infected with JSRV. Retrovirology 2006, 3, 94–108.
23) Dagleish MP, Benavides J Chianini F: Immunohistochemical diagnosis of infectious diseases of sheep. Small Rumin. Res. 2010, 92, 19–35.
24) McNeilly TN, Tennant P, Luján L, Pérez M, Harkiss GD: Differential infection efficiencies of peripheral lung and tracheal tissues in sheep infected with Visna/maedi virus via the respiratory tract. J. Gen. Virol. 2007, 88, 670–679.
25) Cousens C, Gibson L, Finlayson J, Pritchard I, Dagleish MP: Prevalence of ovine pulmonary adenocarcinoma (Jaagsiekte) in a UK slaughterhouse sheep study. Vet. Rec. 2015, 176, 413.
26) Mackay JMK, Nisbet DI: Jaagsiekte – a hazard of intensified sheep husbandry. Vet. Rec. 1966, 78, 18–20.
27) Salvatori D, Gonzalez L, Dewar P, Cousens C, De las Heras M, Dalziel RB, Sharp JM: Successful induction of ovine pulmonary adenocarcinoma in lambs of different ages and detection of viraemia during the preclinical period. J. Gen. Virol. 2004, 85, 3319–3324.
28) Voigt K, Krämer U, Brügmann M, Dewar P, Sharp JM, Ganter M: Eradication of ovine pulmonary adenocarcinoma by motherless rearing of lambs. Vet. Rec. 2007, 161, 129–132.